INDICATIONS OF RELATIONSHIPS BETWEEN PORIFERAN CLASSES USING FULL-LENGTH 18S rRNA GENE SEQUENCES

CHRISTI L. ADAMS, JAMES O. McINERNEY AND MICHELLE KELLY

Adams, C.L., McInerney, J.O. & Kelly, M. 1999 06 30: Indications of relationships between poriferan classes using full-length 18s rRNA gene sequences. *Memoirs of the Queensland Museum* **44**: 33-43. Brisbane. ISSN 0079-8835.

Porifera is traditionally viewed as monophyletic, yet recent molecular data indicate it may be paraphyletic or even polyphyletic. In this study full-length 18S rRNA sequences were derived from two hexactinellid sponges (Class Hexactinellida), four demosponges (Class Demospongiae) and one calcareous sponge (Class Calcarea), in order to test the evolutionary hypotheses of relationship between them, and ultimately, to test the monophyly of Porifera. Phylogenetic analyses yielded congruent polyphyletic topologies with Demospongiae and Hexactinellida, forming a well-supported clade, which excluded the Calcarea. The Calcarea was hypothesised to be more closely related to other diploblasts, forming a clade with the comb-jellies (Phylum Ctenophora). The Kishino-Hasegawa test was applied to explore alternative evolutionary relationships between the sponge classes. Constraining the Calcarea as sister taxon to either Demospongiae or Hexactinellida was rejected in this test, although a monophyletic sponge phylum could not be rejected using this dataset. ❒ *Porifera, Demospongiae, Hexactinellida, Calcarea, molecular phylogeny, evolution, 18S rRNA.*

Christi L. Adams, Queensland Museum, P.O. Box 3300, South Brisbane 4101; James O. McInerney & Michelle Kelly (email: m.kelly@ niwa.cri.nz), Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK; *Present address: National Institute of Water & Atmospheric Research, Private Bag 109-695, Newmarket, Auckland;* *7 May 1999.*

Sponges are regarded as the most primitive multicellular animals, primarily due to their 'simple' body plans and evidence from the fossil record, which together suggest they were the earliest lineage to diverge within the Metazoa. Sponges have attained a 'multicellular' grade of construction, with no development of tissues or organs, and their fossil record extends for at least 600 million years. Due to their shared possession of unique choanocyte cells, the Porifera is generally considered to be a monophyletic phylum. Recent ultrastructural and molecular evidence, however, suggests they may be a paraphyletic or even polyphyletic group of animals (Mackie & Singula, 1983; Reiswig & Mackie, 1983; Cavalier-Smith et al., 1996; Borchiellini et al., 1998; Kruse et al., 1998).

Phylum Porifera, as it is presently recognised, consists of three classes; Demospongiae, Hexactinellida and Calcarea, differentiated primarily by differences in composition and geometry of skeletal components and cellular organisation of the soft parts. Hexactinellida and Demospongiae are characterised, in part, by the common possession of inorganic skeletons composed of siliceous spicules, although some demosponges (Dictyoceratida, Dendroceratida and Verongida), have skeletons composed of only proteinaceous (spongin) fibres and collagen fibrils. Demospongiae and Hexactinellida differ in that the former have spicules with one to four rays (monactine to tetractine), whereas the latter always have triactine or triaxial-derived (pentactinal and hexactinal) spicules. In contrast, Calcarea include sponges with calcium carbonate spicules in the form of calcite. Calcarea and Demospongiae both have representative species of 'coralline sponges' possessing a calcareous aragonitic base, in addition to calcareous or siliceous spicules, respectively. These sponges, now referred to as 'hypercalcified', formerly comprised the Class Sclerospongiae, whereas it is now believed that this grade of construction has evolved independently in different lineages within the two classes (Vacelet, 1985; Reitner, 1992).

Demospongiae and Calcarea have three major cellular layers. The first layer, the pinacoderm, lines all external surfaces of the sponge and is composed of a single layer of pinacocyte cells. The second layer is the choanoderm, composed of choanocytes which are the collared cells that draw water, and hence nutrition, into the sponge via the aquiferous canal system. Lastly, is the mesohyl, a proteinaceous matrix lying between

the pinacoderm and choanoderm, where the skeletal material is found with all other cell types.

Morphologically, Hexactinellida are considerably different from Demospongiae and Calcarea, with syncytial cellular organisation. Instead of pinacocytes, these sponges have a syncytial surface dermal membrane which is contiguous with an inner trabecular membrane that drapes through the sponge interior. The mesolamella in hexactinellids is equivalent to the mesohyl in other sponges (Mackie & Singula, 1983). The mesolamella is composed of collagenous sheets which form a suspensory network for attachment and support of trabecular tissues (Reiswig & Mackie, 1983). Hexactinellids do not possess a choanoderm as in the other two classes, but have a choanosyncytium composed of numerous collared bodies sharing a common nucleus and joined by stoloniferous cytoplasmic bridges. Hexactinellids also possess a unique secondary suspensory network that supports the collars of the collar bodies, which is not present in Demospongiae or Calcarea (Reiswig & Mackie, 1983). Because of these major differences Bergquist (1978) and Reiswig & Mackie (1983) proposed separate phylum and subphylum status, respectively, for the Hexactinellida.

Two major hypotheses have been developed explaining the evolutionary relationships between the sponge classes (Fig. 1). The first suggests that Demospongiae and Hexactinellida are more closely related to each other than to Calcarea, based on their respective similarities in the chemical composition of spicules (Möhn, 1984; Böger, 1988; Ax, 1996). Calcareous spicules are formed extracellularly by several sclerocytes (Ledger & Jones, 1977), whereas siliceous spicules of Demospongiae originate intracellularly within a single sclerocyte, and those of Hexactinellida are formed intrasyncytially by a 'scleroblast mass' containing many nuclei. The central axial filaments of spicules from Demospongiae and Hexactinellida differ in their cross sectional geometry (hexagonal and triangular vs. square, respectively), while calcareous spicules are devoid of a central filament. These differences in the axial filament could be indicative of differences in chemical composition (Reitner & Mehl, 1996). Most authors believe that spicules were derived independently in each of the three classes, and consequently are not useful as phylogenetic character at the class level except to show that spiculogenesis is not homologous in each class.

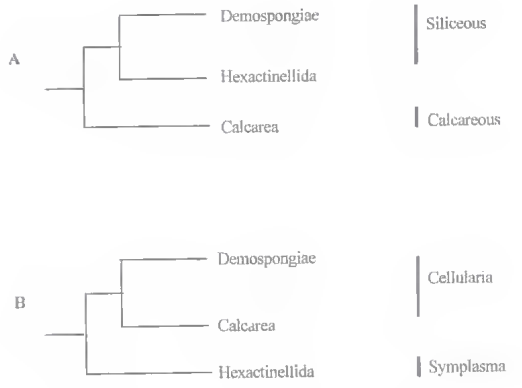

FIG. 1. Diagrammatic representation of the two major hypotheses of relationship between the three poriferan classes. A, Class Demospongiae and Class Hexactinellida are more closely related to each other than to the Calcarea based on chemical composition of the spicules (after Möhn, 1984; Böger, 1988; Ax, 1996). B, Class Calcarea and Class Demospongiae (Subphylum Cellularia) are more closely related to each other than to the Hexactinellida (Subphylum Symplasma) due to their differing cellular condition (after Reiswig & Mackie, 1983).

The second hypothesis proposes that Calcarea and Demospongiae are more closely related to each other than to Hexactinellida, emphasising major differences in cellular condition between these groups. It was proposed that the Subphyla Symplasma (representing hexactinellids) and Cellularia (demosponges and calcareans) be erected to distinguish between these groups (Reiswig & Mackie, 1983). Reitner & Mehl (1996) proposed the term Pinacophora, stating that it was more appropriate than the term Cellularia when comparing sponges with other metazoans. They identified three apomorphies for the group which separated it from Hexactinellida: 1) the presence of a pinacoderm, 2) ball-shaped choanocyte chambers in the adult, and 3) the ability to produce a calcareous ('hypercalcified') basal skeleton, as in the coralline sponges (Reitner & Mehl, 1996).

Hexactinellida first appeared in the fossil record during the Late Proterozoic, approximately 540my ago, whereas Calcarea and Demospongiae did not appear until some 50my later, in the Early Cambrian (Finks, 1970). It has since been proposed, however, that precursors to extant sponges were devoid of spicules and therefore would not have fossilised easily (Vacelet, 1985), which lends apparent support to the second hypothesis. Moreover, the known fossil history for

sponges has recently been challenged by Li et al. (1998), who claim to have found fossil demosponges 580my old. They suggest that demosponges were the first class to evolve, rather than hexactinellids (Li et al. 1998), supporting the first hypothesis.

As demonstrated by Van Soest (1987), sponge classification at the higher levels is problematic due to a lack of synapomorphic characters and numerous assumed homoplasies. Characters such as spicule composition and cellular construction may be valid and truly indicative of phylogeny, but it is difficult to determine which character should be given more weight, if at all.

Recent molecular studies have been very helpful in providing additional characters to assist with phylogenetic reconstructions. In an attempt to elucidate the phylogenetic history for Porifera, various gene sequences have been explored including the small subunit of the ribosomal 18S gene (18S rRNA), heat shock protein 70 (Hsp70) and Protein Kinase C.

Cavalier-Smith et al. (1996) analysed full-length 18S rRNA genes which yielded a paraphyletic Porifera. A Demospongiae- Hexactinellida clade formed a sister group to a Calcarea-Ctenophora clade. This suggests that Demospongiae and Hexactinellida are more closely related to each other than they are to Calcarea. This hypothesis contradicts the proposal for subphylum status for Hexactinellida, as suggested by Reiswig & Mackie (1983) and Reitner & Mehl (1996).

Analysis of the two protein coding genes, Hsp70 (Borchiellini et al., 1998) and Protein Kinase C (Kruse et al., 1998) produced a polyphyletic and paraphyletic Porifera, respectively. Koziol et al. (1997) also examined the Hsp70 gene, but considered it to be too conservative for resolution within the Porifera. Borchiellini et al. (1998), however, examined the Hsp70 gene using the first and second codon positions and found, with low bootstrap support, that Calcarea and Demospongiae formed a clade to the exclusion of Hexactinellida. Their results are controversial in that sponges were shown to be a group derived from other Metazoa. Cnidarians were hypothesised as being the first metazoans to diverge, followed by Ctenophora which formed a sister group to sponges. In some analyses, hexactinellids formed a clade with ctenophores, rather than with other sponge groups, while the 18S rRNA gene showed ctenophores to be more closely related to Calcarea (Cavalier-Smith et al., 1996). Analyses of the Hsp70 gene always resulted in a Demospongiae-Calcarea clade, which supports the concept of the Subphylum Cellularia.

Results from analysis of the Protein Kinase C gene were more similar to the 18S rRNA data, with Calcarea forming a clade with the lower metazoans. These data also showed that hexactinellids were the first to diverge from the metazoans, while demosponges formed a sister group to a calcarean-metazoan clade (Kruse et al, 1998). Hexactinellida did not form a clade with demosponges, but instead formed a sister group to all other metazoans. Unfortunately, analyses for each gene yielded differing topologies, each with low bootstrap support, leaving the alleged phylogenetic relationships of sponges unresolved. Even though these studies yielded conflicting phylogenetic patterns, it is still possible that Porifera may not be monophyletic, as traditionally believed.

To date, these are the only molecular studies which have included representatives from each of the three classes of sponges (although the only hexactinellid 18S rRNA sequence has not yet been made available in GenBank; West & Powers, 1993). The 18S rRNA gene is the most extensively studied gene, with the largest database available for comparison. Universal primers have also been developed, making this gene relatively easy to obtain sequences in a short period of time. Although it has been suggested that the 18S rRNA gene does not have enough signal to address the phylogenetic history of the lower metazoans, due to over saturation (Rodrigo et al., 1994), it has been demonstrated that increased taxon sampling can assist in resolving phylogenetic relationships, principally by spreading homoplasic signal among a greater diversity of internal branches (Hillis, 1996).

The aims of this study were to test the monophyly of Porifera by examining relationships between Demospongiae, Calcarea and Hexactinellida. In addition to those six sequences already available in GenBank, we generated six additional full-length 18S rRNA sequences, strengthening inference from all available data. Phylogenetic trees inferred from Distance Matrix (DM), Maximum Likelihood (ML) and Maximum Parsimony (MP) methods were compared with hypotheses generated from other genes, utilising Constraint Analysis via the Kishino-Hasegawa test (Kishino & Hasegawa, 1989).

TABLE 1. List of sponges used in this study with corresponding museum voucher number and collection locality (identified by MK).

Classification	Voucher number	Collection locality
Class Demospongiae		
Subclass Tetractinomorpha		
Vetulina stalactites Schmidt, 1879	BMNH 1998.3.19.1	Caribbean Sea
Acanthochaetetes wellsi Hartman & Goreau, 1975	BMNH 1995.11.2.2	Palau, Micronesia
Subclass Ceractinomorpha		
Clathria (Thalysias) reinwardti Vosmaer, 1880	MKB 142	Pohnpei, Micronesia
Negombata corticata (Carter, 1879)	BMNH 1998.3.19.2	Red Sea
Class Hexactinellida		
Subclass Hexasterophora		
Sympagella nux Schmidt, 1870	HBOM 003:00925	Turks & Caicos
Margaritella coeloptychioides Schmidt, 1880	HBOM 003:00929	Turks & Caicos
Class Calcarea		
Subclass Calcinea		
Leucetta sp.	HBOM 27:X:96:3:305	Bahamas

MATERIALS AND METHODS

SAMPLE COLLECTION AND SELECTION. Specimens were collected by SCUBA and manned-submersible from various localities between 1989-1996 (Table 1). Immediately upon collection a small piece of the sponge, approximately $3cm^3$, was removed from the interior with a clean scalpel blade to minimise surface epibiont contamination. This voucher was either frozen at -20°C or diced as finely with a sterile razor blade and immediately placed in Guanidium Chloride (GnCl) Buffer [6M GnCl, 5% Tween 20, 0.5% Triton X-100 in 1L Tris-EDTA buffer, pH 8.0 (100mM Tris, 30mM EDTA)] for stable storage of the lysed cells and DNA. A representative piece of the sponge was placed in 70% ethanol for subsequent taxonomic identification. Voucher specimens of all taxa were deposited at The Natural History Museum, London (BMNH), the Harbor Branch Oceanographic Institution Museum, Fort Pierce, Florida (HBOM) and in the personal collection (MKB) of MK.

EXPERIMENTAL PROCEDURES. Sponge cell buffered lysate was diluted four-fold with autoclaved analar H_2O, and frozen specimens were thawed and ground with a micropestle to form a slurry prior to DNA extraction, using standard phenol:chloroform extraction procedures (Sambrook et al., 1989). Full-length (approximately 1300bps) 18S rDNA was amplified with the forward and reverse primers 18Sf20 and 18Sr21, respectively (McInerney et al., in press). PCR conditions for 1.6ng/µl DNA in 50µl reactions were: initial denaturation at 94°C for 5mins, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min, and extension at 72°C for 1min. The product was electrophoresed on an 0.8% agarose gel stained with 1µg/µl ethidium bromide to check band size, and then purified from the gel with the Qiaex II PCR purification kit (Qiagen Ltd, UK), following manufacturers instructions. In addition to the two PCR primers, eight internal primers were used to sequence both chains automatically, utilising the dideoxy chain termination method (Sanger et al., 1977) (forward primers 377F CCGGAGARGGAGCCTGA, 577F GCCAGC MGCCGCGGT, 1262F GGTGGTCGATG GCCG and 1510F CAGGT CTGTGATGCC and their complementary reverse primers called 377R, 577R, 1262R and 1510R). Each contiguous sequence fragment was replicated with at least one overlapping fragment (Amersham Cycle Sequencing Kit). Ambiguous nucleotide positions were coded according to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature. Sequences were managed utilising Sequencher 3.0 software (Gene Codes Corporation, 1995). New sequences were deposited in the GenBank sequence repository (http://www2.ncbi.nlm.nih.gov) under accession numbers *Vetulina stalactites* (AF084236), *Acanthochaetetes wellsi* (AF084237), *Clathria (Thalysias) reinwardti* (AF084238), *Negombata corticata* (AF084239), *Sympagella nux* (AJ224123), *Margaritella coeloptychioides* (AJ224124) and *Leucetta* sp. (AF084240).

PHYLOGENETIC RECONSTRUCTION. To test the monophyly of Porifera, sequences from the lower metazoans were included in the analysis: Phylum Porifera - *Tetilla japonica* (D15067), *Microciona prolifera* (L10825) (which Hooper (1996) referred to the subgenus *Clathria (Clathria)* based on taxonomic re-evaluation of type material, and we assume that GenBank L10825 belongs to this species), *Axinella polypoides* (U43190), *Clathrina cerebrum* (U42452), *Scypha ciliata* (L10827) (which belongs to *Sycon*, follwing Dendy & Row, 1913; Gert Woerheide, pers.comm.), *Sycon calcaravis* (D15066); Phylum Placozoa -

Trichoplax adhaerens (L10828); Phylum Ctenophora - *Beroe cucumis* (D15068), *Mnemiopsis leidyi* (L10826); Phylum Cnidaria - *Anemonia sulcata* (X53498), *Tripedalia cystophora* (L10829). Representatives of each of the major fungal groups were chosen as the outgroup taxon, due to their inferred position as a sister group to Metazoa, according to rRNA data (Wainright et al., 1993) and protein data (Baldauf & Palmer, 1993): Fungi - *Aureobasidium pullulans* (M55639), *Saccharomyces cerevisiae* (Z75578), *Athelia bombacina* (M55638), *Blastocladiella emersonii* (X54264). Taxa selected for this study were retrieved from a secondary structure alignment maintained on the Ribosomal Database Project (RDP) database (http://rdpwww.life.uiuc.edu/ index2.html; Maidak et al., 1996). The profile alignment option of ClustalW was then used to combine the two alignments (Higgins & Sharp, 1988). Sequences were aligned using ClustalW 1.7 and then modified by eye in the Genetic Data Environment, GDE, (Smith et al., 1994) on a SUN workstation. A conservative approach was used for alignment. Only those positions whose alignment was ambiguous were chosen for analysis, thus ruling out a significant number of potential positions. Approximately 1300bps were sequenced. The new alignment (including other sponges, metazoans and fungi), was 2590bps in length, and after removal of ambigous positions the resulting alignment was 987bps long.

Phylogenetic hypotheses were constructed, and sequence statistics were evaluated, using PAUP* 4.0.0d64 test version (Swofford, in press). The likelihood ratio test statistic was used to evaluate which evolution model fit the data best (Goldman, 1993). This was achieved by first constructing a neighbour joining tree calculated by the Jukes & Cantor (1969) method. Using this guide tree and maximum likelihood criteria, the transition-transversion ratio, base composition and proportion of invariable sites were estimated using the Newton-Raphson method implemented in Paup*4.0. Each of these variables were calculated separately, and then entered into the model to calculate the next variable. This process was repeated until the best maximum likelihood value was reached. The chosen evolutionary model was that which yielded the optimal likelihood value without compromising model complexity. The reliability of internal branches was evaluated by the bootstrap resampling method (Felsenstein, 1985). In each analysis, 100 iterations were carried out for each optimality criterion. A 50% majority rule consensus tree was inferred from the resulting bootstrap partition table.

The inferred phylogenetic relationships derived from the new 18S rRNA sequences were compared with the major phylogenetic hypotheses derived from morphological characters and other genes. Using MacClade (Maddison & Maddison, 1992), we constructed trees representing competing hypotheses. The constrained trees were examined using the Kishino-Hasegawa test (Kishino & Hasegawa, 1989), for both ML and MP methods.

ABBREVIATIONS. DM, Distance Matrix; ML, Maximum Likelihood; MP, Maximum Parsimony methods.

RESULTS

Prior to bootstrapping, MP, DM and ML methods yielded trees with virtually identical topologies, except for the branch arrangement within Demospongiae which was largely unresolved. For the DM method, the F84 model was used (Felsenstein, 1984), with the minimum evolution objective function. The proportion of sites assumed to be invariable = 0.686332. For the MP method, (parsimony informative sites = 155), the characters were treated as unordered and equally weighted. The ML analysis was conducted with a Two-type substitution model with an estimated transition/transversion ratio of 1.553481, estimated base frequencies of A = 0.270333, C = 0.206219, G = 0.258425 and T = 0.265023, and an estimated proportion of invariable sites = 0.686760 (with equal rates of variation for all sites). Using the MP method as representative, two equally parsimonious trees with minimal differences in tree topology were recovered (CI = 0.669, RI = 0.723, tree length = 465, total number of characters = 987, parsimony informative characters = 155) (Fig. 2). The only difference in branch arrangement between the two MP trees was the position of *Acanthochaetetes* within the Demospongiae. In Tree A (Fig. 2), *Acanthochaetetes* is the earliest separation within Tetractinomorpha, whereas in Tree B (Fig. 2), *Acanthochaetetes* is the earliest separation of the Ceractinomorpha clade. The three sponge classes are monophyletic in both trees, with Demospongiae as sister taxon to Hexactinellida, while Calcarea formed a sister taxon to Ctenophora.

Within Demospongiae, Subclass Tetractinomorpha is represented by *Vetulina* and *Tetilla*,

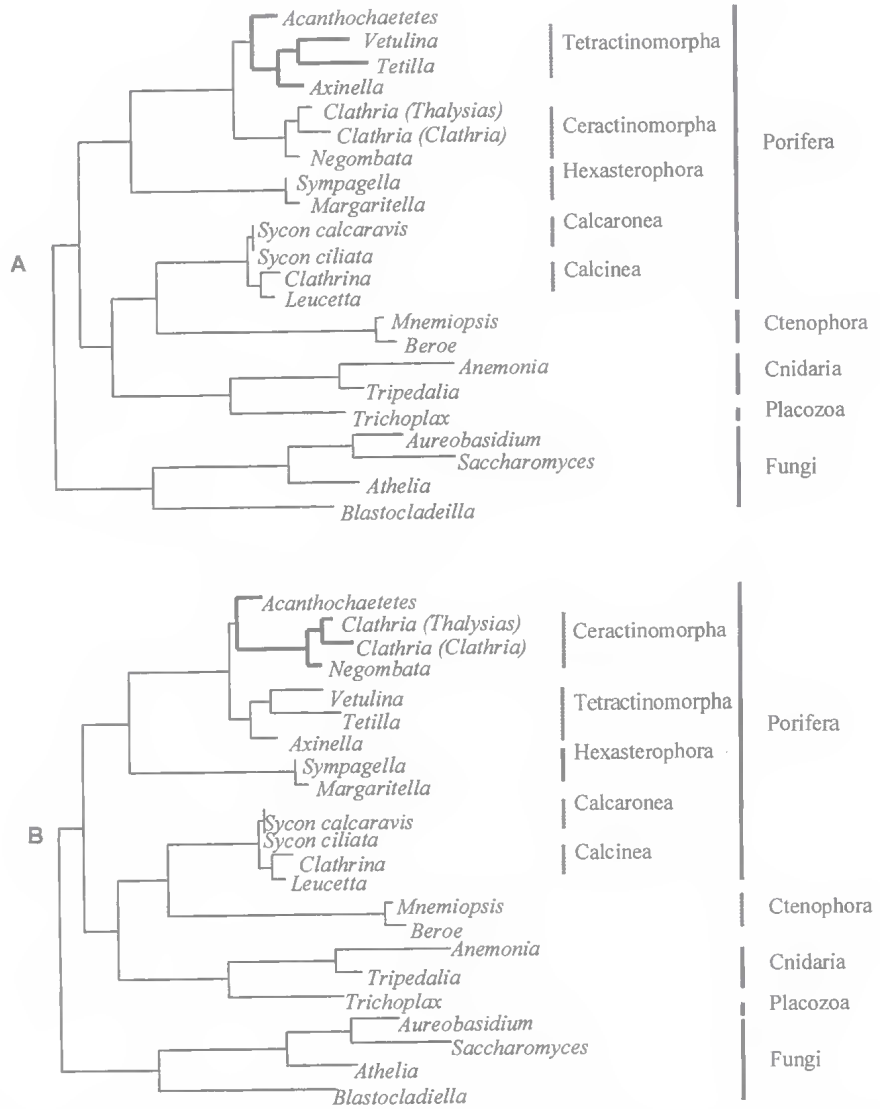

FIG. 2. Two most parsimonious trees derived from molecular data analysed using the assumptions of MP (CI = 0.669 , RI = 0.723, tree length = 465, total number of characters = 987, parsimony informative characters = 155). A, *Acanthochaetetes* is the earliest taxon to diverge from the Tetractinomorpha. B, *Acanthochaetetes* is the earliest taxon to diverge from the Ceractinomorpha.

which form a common clade. *Axinella* was also located within the tetractinomorphan clade. The family Axinellidae was formerly considered to be a tetractinomorph on the basis of their reproductive strategies, but Van Soest et al. (1990) suggested that, based on morphology, there was more support for their placement among Ceractinomorpha. Our dataset, however, places Axinellidae (represented by *Axinella*), in Tetractinomorpha. *Acanthochaetetes* is also traditionally recognised as a tetractinomorph sponge, but this position is unstable, grouping with tetractinomorphan Tree A, and with Ceractinomorpha in Tree B. Ceractinomorpha were represented by two species of *Clathria,* and *Negombata*, where the latter taxon has had a questionable affinity with switches between Tetractinomorpha (Bergquist, 1978) and Ceractinomorpha (Topsent, 1922). Kelly-Borges & Vacelet (1995) suggested that based on morphology, chemistry and reproduction, *Negombata* has a closer affinity to Ceractinomorpha, Order

TABLE 2. Results of the K-H test for alternatives to the MP/ML tree. Tree number 1 is the best tree in both analyses. Tree 2 is a constrained tree that retains a clade containing all of the sponge taxa. Tree 3 is a constrained tree that places Calcarea with Demosponges to the exclusion of all other taxa. Tree 4 places Calcarea with Hexactinellida. The second column gives the ML score (negative log-likelihood) for each tree. The third column gives the difference in Log-likelihood between alternatives. The fourth column indicated whether or not an alternative is significantly worse than the optimal tree (P<0.05). The fifth column gives the tree length of the various trees. Column number six gives the difference in tree length between alternative trees. The last column indicates the significance of the result (an asterisk indicates a P-value < 0.05).

Tree	Likelihood			Parsimony		
	-Ln L	Diff -ln L	P*	Length	Diff.	P*
1	3960.28210	(best)		466	(best)	
2	3964.75361	4.47151	0.4971	469	3	0.3176
3	4128.96432	168.68222	<0.0001*	514	48	<0.0001*
4	4122.38473	162.10263	<0.0001*	510	44	<0.0001*

Poecilosclerida. Our data lend support to this hypothesis.

There were only two representative taxa for Hexactinellida, each belonging to the same subclass, Hexasterophora, forming a monophyletic clade in all three analyses. Hexactinellida consistently form a sister group with Demospongiae.

The topology of trees obtained from the three methods of analysis varied only slightly from that of the DM tree, recovering a topology which suggests that the calcareous Subclass Calcaronea arose from within the Subclass Calcinea (not shown). Both MP and ML methods yielded a topology with the two calcareous subclasses positioned as sister taxa. Our data show the Subclasses Calcaronea and Calcinea to be valid groupings, but at least three taxa per group are needed to infer relationships. Calcarea form a sister group relationship with Ctenophora. This clade is hypothesised to be derived from the diploblastic animals (Cnidaria and Placozoa).

A total of 100 bootstrap replicates were constructed for each of the three methods of analysis; DM, MP and ML (Fig. 3). Each of the three classes is consistently recovered, with high bootstrap support as a monophyletic class. Hexactinellida formed a clade with 100% bootstrap support for each method, and Demospongiae formed a monophyletic group with bootstrap values of 100, 99 and 88 for DM, MP and ML methods, respectively. There were no representatives of the Subclass Homoscleromorpha in these analyses due to difficulties we encountered during PCR. The topology of trees relating most Demospongiae taxa received less than 50% bootstrap support. The *Negombata - Clathria* clade was the only relationship that remained intact and formed a monophyletic group, with bootstrap values of 98 for DM, 100 for MP and 99 for ML, yielding further support to the hypothesis that *Negombata* is more closely related to Poecilosclerida than to the tetractinomorph Hadromerida.

Support for a Demospongiae-Hexactinellida clade was relatively high (DM 89%, MP 81% and ML 70%), while Calcarea formed a monophyletic group with bootstrap support of 100 for the DM and MP trees and 98 for the ML tree. Calcarea consistently formed a sister taxon with Ctenophora, although there was only low bootstrap support of 64, 55 and 56, for DM, MP and ML methods, respectively.

In order to test alternative hypotheses on possible relationships between the sponge classes, the Kishino-Hasegawa (K-H) test was employed for both the MP and ML methods. Constraint trees were designed and compared against the optimal tree inferred from our data, which showed Calcarea was more closely related to Ctenophora, and Demospongiae and Hexactinellida formed a sister-group. The optimal tree for each constraint analysis was found using the MP and ML methods, and then the resulting tree was compared with the optimal, unconstrained tree to test for significant differences (Table 2).

For the K-H test using ML and MP methods, three constrained trees were designed and tested against the optimal tree derived from the molecular data (Tree 1): a monophyletic clade containing all three classes (Tree 2); a clade containing the Hexactinellida as a sister taxon to a Demospongiae-Calcarea clade (Tree 3); and a clade containing the Demospongiae as a sister taxon to a Hexactinellida-Calcarea clade (Tree 4). All alternative trees were rejected when compared to the optimal tree, with the exception of a monophyletic Porifera (Tree 2).

The optimal tree from ML analysis has a log-likelihood value of -3960.28210, while the best tree which retained a monophyletic sponge clade had a log-likelihood score of -3964.75361. A two-tailed T-test did not reject this hypothesis as

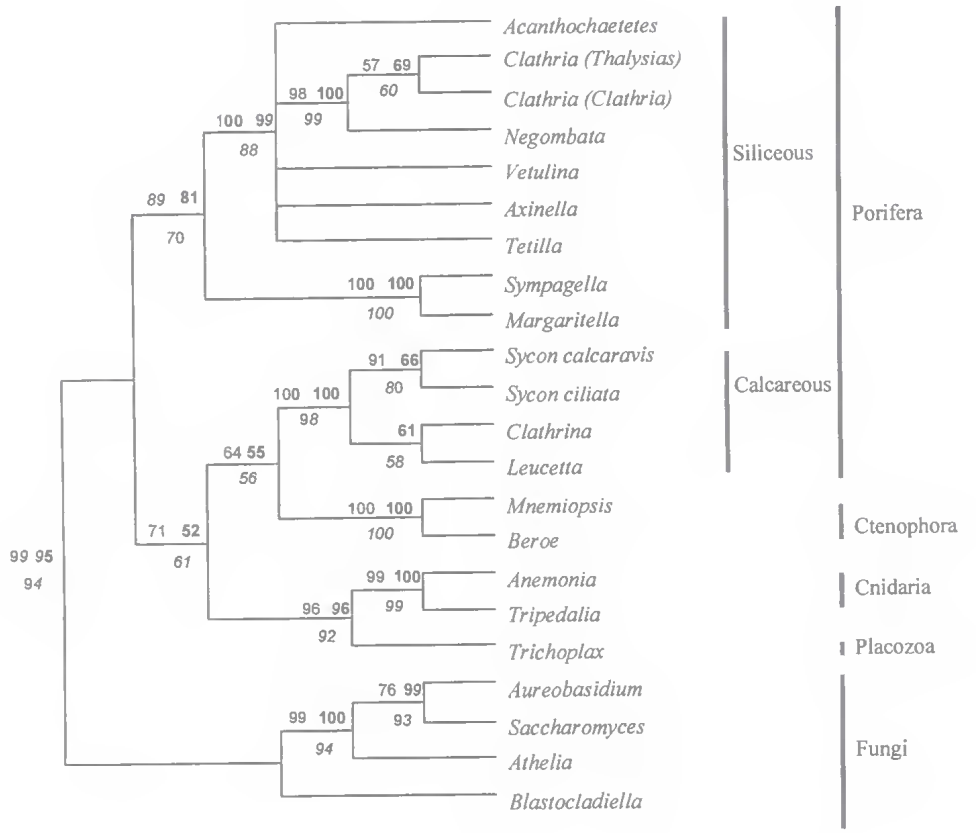

FIG. 3. Phylogenetic consensus tree for DM, MP and ML with bootstrap values corresponding to each method. 50% majority rule consensus trees are displayed. DM values are presented in normal text, MP values are in bold and ML bootstrap values are in italics.

being significantly worse than the ML tree. Similarly, using the MP analysis method, the most parsimonious tree was only three steps shorter than a tree which contained a monophyletic sponge clade, and the Log-likelihood of a monophyletic tree was only 0.1% less likely than the optimal tree. In both instances, the alternative hypothesis was not significantly worse than the optimal tree ($P<0.05$). Given that we cannot reject the alternative hypothesis, it would be unwise to suggest that the phylum is not monophyletic.

DISCUSSION

Bootstrap results indicate a polyphyletic arrangement for Phylum Porifera, and support the theory that siliceous sponges evolved separately from calcareous sponges. This arrangement has previously been suggested in the literature, supported by data from 18S rRNA (Kobayashi et al., 1993; Cavalier-Smith et al., 1996), 28S rRNA (Lafay et al., 1992) and Protein Kinase C genes (Kruse et al., 1996). In these earlier studies, the sponge classes and subclasses were not extensively represented in analyses, whereas our data doubles the number of sponge 18S rRNA sequences analysed, and yet yields the same topology. Our results also show that calcareous sponges are derived from other metazoans, which are generally considered to have evolved later than the siliceous sponges, based on morphological data and the fossil record.

The poriferan classes have several apparent apomorphies, but upon closer examination these may be convergent characters. Differences in spicule geometry and spiculogenesis are obvious between calcareous and siliceous sponges, but possibly also between the Hexactinellida and Demospongiae. There are notable differences in choanocyte size, shape and arrangement within choanocyte chambers at the class and order

levels. While species of Demospongiae and Calcarea share similar cellular construction and mesohyl characteristics, when compared to Hexactinellida, Calcarea lack the collagen and proteinaceous fibre development found in Demospongiae. Larval morphology is also very different within and between the three classes. It is possible that convergent evolution has taken place between these three classes due to a common benthic, filter-feeding lifestyle.

It is also quite difficult to explain why Calcarea are consistently grouped with the Ctenophora according to molecular data, even with low bootstrap support. Morphologically, Ctenophora are much more complex than Porifera, with true tissue structure such as mesodermal muscle, gonoducts, and an anal pore; however increased complexity does not necessarily equate with a more derived evolutionary state. It is conceivable that Calcarea have secondarily lost characters that are present in ctenophores. Cavalier-Smith et al. (1996) suggest that calcareous spicules found in Calcarea may be homologous to those found in Cnidaria. A loss of spicules could have occurred in Ctenophora. They also suggest that larvae found in the subclass Calcinea (Calcarea) are morphologically much more similar to those of other animals than they are to other sponges. Additionally, the position of ctenophores with respect to other diploblasts, based on molecular data, rests on analysis of only two representatives of the phylum.

Although we failed to confirm whether the Porifera was a monophyletic taxon using our expanded molecular dataset, the hypothesis of poriferan monophyly is not rejected based on molecular data. The exact placement of calcareous sponges is problematic and requires further empirical support from sequences derived from a greater diversity of sponges and ctenophorans. Surprisingly, when ctenophores were removed from analyses (data not shown), Calcarea remained a sister taxon to other diploblasts, and not with siliceous sponges (Demospongiae, Hexactinellida). Although these results are congruent with previous data, they are difficult to explain on the basis of 'classical' morphological characters and the paleontological record.

Using the bootstrap resampling method, we have shown that a partition separating the Classes Hexactinellida and Demospongiae from all other taxa is reasonably well supported. Although bootstraps are not statistically high, any alternative arrangement receives very little support. This arrangement does, however, refute the hypothesis that Hexactinellida merit phylum or subphylum status. The conclusions of this study do not rule out the monophyletic nature of the phylum. Until data are gathered that can yield high confidence levels for a polyphyletic phylum, the possibility of sponges being monophyletic cannot be dismissed.

ACKNOWLEDGEMENTS

The authors wish to thank the Division of Biomedical Marine Research, Harbor Branch Oceanographic Institution, and the crews of the R.V. 'Seward Johnson' and R.V. 'Edwin Link' for logistic, field and laboratory support for the collection of material. We thank Ms Efrat Meroz, Tel Aviv University, for providing a specimen of *Negombata*, and the Coral Reef Research Foundation for supporting the collection of *Clathria (Thalysias) reinwardti*. The authors thank Klaus Borges for assistance with histological preparations of Demospongiae, Dr Henry Reiswig for identification of Hexactinellida, and Dr Franz Lang for DNA preparation of Hexactinellida. We are grateful to Dr David Swofford for providing a pre-release of PAUP*4.0. This research was funded primarily by a Natural History Museum, London, PhD fellowship to CA. CA also thanks the Queensland Museum and sponsors of the 5th ISS for funding to attend the Brisbane Symposium, to present this paper to international peers. MK and JMcI thank the Biological and Biotechnological Research Council, UK and British Airways for field support that contributed to this research. This is a contribution to the Coral Reef Research Foundation, Micronesia. This is Harbor Branch Oceanographic Institution Contribution 1282.

LITERATURE CITED

AX, P. 1996. Multicellular animals: A new approach to the phylogenetic order in nature. (Springer-Verlag: New York).

BALDAUF, S.L. & PALMER, J.D. 1993. Animals and fungi are each other's closest relatives: congruent evidence from multiple proteins. Proceedings of the National Academy of Science USA 90: 11558-11562.

BERGQUIST, P.R. 1978. Sponges. (Hutchinson: London).

BÖGER, H. 1988. Versuch über das phylogenetische system der Porifera. Meyniana 40: 143-154.

BORCHIELLINI, C., BOURY-ESNAULT, N., VACELET, J. & LE PARCO, Y. 1998. Phylogenetic analysis of the Hsp70 sequences

reveals the monophyly of metazoa and specific phylogenetic relationships between animals and fungi. Molecular Biology and Evolution 15(6): 647-655.

BOROJEVIC, R., BOURY-ESNAULT, N. & VACELET, J. 1990. A revision of the supraspecific classification of the subclass Calcinea (Porifera, class Calcarea). Bulletin of the Museum of Natural History, Paris (4) 12(2): 243-276.

CAVALIER-SMITH, T., ALLSOPP, M., CHAO, E., BOURY-ESNAULT, N. & VACELET, J. 1996. Sponge phylogeny, animal monophyly, and the origin of the nervous system: 18S rRNA evidence. Canadian Journal of Zoology 74: 2031-2045.

CHOMBARD, C., BOURY-ESNAULT, N., TILLIER, A. & VACELET, J. 1997. Polyphyly of "Sclerosponges" (Porifera, Demospongiae) supported by 28S ribosomal sequences. Biological Bulletin 193: 359-367.

DENDY, A. & ROW, R.W.H. 1913. The classification and phylogeny of the calcareous sponges; with a reference list of all the described species, systematically arranged. Proceedings of the Zoological Society of London (1913): 704-813.

FELSENSTEIN, J. 1984. Distance methods inferring phylogenies: a justification. Evolution 38:16-24.

1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

FINKS, R.M. 1970. The evolution and ecological history of sponges during Paleozoic times. Pp. 3-22. In Fry, W.G. (ed.) Biology of the Porifera. Symposium of Zoological Society of London. No. 25. (Academic Press: London).

GOLDMAN, N. 1993. Statistical tests of models of DNA substitution. Journal of Molecular Evolution 36: 182-198.

HILLIS, D.M. 1996. Inferring complex phylogenies. Nature 12 (383, 6596): 130-131.

HOOPER, J.N.A. 1996. Revision of Microcionidae (Porifera: Poecilosclerida: Demospongiae), with description of Australian species. Memoirs of the Queensland Museum 40: 1-626.

JUKES, T.H. & CANTOR, C.R. 1969. Evolution of protein molecules. Pp. 21-132. In Munro H.N. (ed.) Mammalian Protein Metabolism. (Academic Press: New York).

KASHINO, H. & HASEGAWA, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of the Hominoidea. Journal of Molecular Evolution 29: 170-179.

KELLY-BORGES, M., BERGQUIST, P.R. & BERGQUIST, P. 1991. Phylogenetic relationships within the order Hadromerida (Porifera, Demospongiae, Tetractinomorpha) as indicated by ribosomal RNA sequence comparisons. Biochemical Systematics and Ecology 19(2): 117-125.

KELLY-BORGES, M. & POMPONI, S. 1994. Phylogeny and classification of lithistid sponges (Porifera: Demospongiae): a preliminary assessment using ribosomal DNA sequence comparisons. Molecular Marine Biology and Biotechnology 3(2): 87-103.

KELLY-BORGES, M. & VACELET, J. 1995. A revision of *Diacarnus* Burton and *Negombata* de Laubenfels (Demospongiae: Latrunculidae) with descriptions of new species from the west central Pacific and the Red Sea. Memoirs of the Queensland Museum 38(2): 477-503.

KOBAYASHI, M., TAKAHASHI, M., WADA, H. & SATOH, N. 1993. Molecular phylogeny inferred from sequences of small subunit ribosomal DNA, supports the monophyly of the metazoa. Zoological Science 10: 827-833.

KOZIOL, C., LEYS, S.P., MÜLLER, I.M. & MÜLLER, W.E.G. 1997. Cloning of Hsp70 genes from the marine sponges *Sycon raphanus* (Calcarea) and *Rhabdocalyptus dawsoni* (Hexactinellida). An approach to solve the phylogeny of sponges. Biological Journal of the Linnean Society 62: 581-592.

KRUSE, M., LEYS, S.P., MÜLLER, I.M. & MÜLLER, W.E.G. 1998. Phylogenetic position of the Hexactinellida within the Phylum Porifera based on the amino acid sequence of the Protein Kinase C from *Rhabdocalyptus dawsoni*. Journal of Molecular Evolution 46: 721-728.

LAFAY, B., BOURY-ESNAULT, N., VACELET, J. & CHRISTEN, R. 1992. An analysis of partial 28S ribosomal RNA sequences suggests early radiations of sponges. BioSystems 28: 139-151.

LEDGER, P. & JONES, W.C. 1977. Spicule formation in the calcareous sponge *Sycon ciliatum*. Cell and Tissue Research 181: 553-567.

LI, C.-W., CHEN, J.-Y. & HUA, T.-E. 1998. Precambrian sponges with cellular structures. Science 279: 879-882.

MACKIE, G. & SINGLA, C. 1983. Studies on hexactinellid sponges. I. Histology of *Rhabdocalyptus dawsoni* (Lambe, 1873). Philosophical Transactions of the Royal Society (London) 301: 365-400.

MADISON, W.P. & MADISON, D.R. 1992. MacClade. Version 3.0 (Sinauer Associates: Sunderland).

MAIDAK, B., OLSEN, J., LARSEN, N., OVERBEEK, R., MCCAUGHEY, M. & WOESE, C. 1996. The Rihosomal Database Project (RDP). Nucleic Acids Research 24(1): 82-85.

McINERNEY, J.O., ADAMS, C.L., BORGES, K.D. & KELLY-BORGES, M. (in press) Phylogenetic resolution within the Phylum Porifera utilising 18S and 28S rRNA genes. Molecular Phylogeny and Evolution.

MÖHN, E. 1984. System und phylongenie der lebewesen Bd-1 Physikalische, chemische und biologische evolution, Prokaryota, Eukaryota (bis Ctenophora). (Schweizerbart: Stuttgart).

MÖHN, E. 1984. System und phylongenie der lebewesen Bd-I Physikalische, chemische und biologische evolution, Prokaryota, Eukaryota (bis Ctenophora). (Schweizerbart: Stuttgart).

REISWIG, H.M. & MACKIE, G.O. 1983. Studies on hexactinellid sponges. III. The taxonomic status of Hexactinellida within the Porifera. Philosophical Transactions of the Royal Society of London, B Biological Science 304:419-428.

REITNER, J. 1992. 'Corraline Spongien'. Der versuch einer phylogenetisch-taxonomischen analyse. Berliner Geowissenschaftliche Abhandlungen (E) I: 1-352.

REITNER, J. & MEHL, D. 1996. Monophyly of the Porifera. Verhandlungen der naturwissenschaftlicht der Hamburg 36: 5-32.

RODRIGO, A., BERGQUIST, P.R. & BERGQUIST, P. 1994. Inadequate support for an evolutionary link between the metazoa and the fungi. Systematic Biology 43(4): 578-584.

SAMBROOK, J., FRISCHT, E. & MANIATIS, T. 1989. Molecular cloning: A laboratory manual. (Cold Spring Harbor Laboratory Press: Cold Spring Harbor).

SANGER, F., NICKLEN, S. & COULSEN, A. 1977. DNA sequencing with chain termination inhibitors. Proceedings of the National Academy of Science USA 74: 5463-5467.

SMITH, S.W., OVERBEEK, R., WOESE, C.R., GILBERT, W. & GILLEVET, P.M. 1994. The Genetic Data Environment: an expandable guide for multiple sequence-analysis. Computer Applications in the Biosciences 10: 671-675.

SOEST, R.W.M. VAN 1987. Phylogenetic exercises with monophyletic groups of sponges Pp. 227-241. In Vacelet, J. & Boury-Esnault, N. (eds) Taxonomy of Porifera. NATO ASI Series. Vol. G13. (Springer-Verlag: Berlin).

SOEST, R.W.M. VAN, DIAZ, M.C. & POMPONI, S.A. 1990. Phylogenetic classification of the Halichondrida (Porifera, Demospongiae). Beaufortia 40: 15-62.

SWOFFORD, D.L., OLSEN, G.J., WADDELL, P.J. & HILLIS, D.M.. 1997. Phylogenetic inference. Pp. 407-514. In Hillis, D.M., Moritz, C. & Mable, B.K. (eds) Molecular Systematics. 2nd Edition. (Sinauer Associates: Sunderland).

SWOFFORD, D.L. (in press). PAUP*: Phylogenetic Analysis Using Parsimony (and other methods). Version 4.0. (Sinauer Press: Massachussetts).

TOPSENT, E. 1922. Les mégasclères polytylotes des Monaxonides et la parenté des Latrunculiines. Bulletin de l' Institut Océanographique de Monaco 415: 1-8.

VACELET, J. 1985. Coralline sponges and the evolution of the Porifera. Pp. 1-13. In Morris, S.C., George, J.D., Gibson, R. & Platt, H.M. (eds) The origins and relationships of lower invertebrates. (Clarendon Press: Oxford).

WAINRIGHT, P.O., HINKLE, G., SOGIN, M.L. & STICKEL, S.K. 1993. Monophyletic origins of the Metazoa: an evolutionary link with fungi. Science 260: 340-342.

WEST, L. & POWERS, D. 1993. Molecular phylogenetic position of hexactinellid sponges in relation to the Protista and Demospongiae. Molecular Marine Biology and Technology 2: 71-75.

ZHARKIKH, A. & LI, W.-H. 1992. Statistical properties of bootstrap estimation of phylogenetic variability from nucleotide sequences. II. Four taxa without a molecular clock. Journal of Molecular Evolution 35: 356-366.

APPROACH TO THE PHYLOGENY OF AXINELLIDAE (PORIFERA: DEMOSPONGIAE) USING MORPHOLOGICAL AND MOLECULAR DATA. *Memoirs of the Queensland Museum 44: 43. 1999:-* A set of 27 species of marine sponges of the Axinellidae and related families was selected with the aim of testing the monophyly of Axinellidae and investigating their phylogenetic relationships using cladistic methods. Partial 28S rDNA sequences, including the D3 domain, and traditional morphological characters were used independently to construct phylogenetic trees. Alignment of the sequences using the appropriate model of secondary structure of the RNA was compared to that produced by the ClustalW. The alignment using secondary structure constraints produced a better estimate of the phylogeny and was demonstrated to be an effective and objective method.

The results from the analyses of the molecular and morphological data sets are not fully congruent; the morphological data suggest that Axinellidae is monophyletic, however the molecular data suggest that it is not monophyletic. In both cases the sampled members of the family are closely related to those of Halichondriidae and Dictyonellidae. Tests of heterogeneity (reciprocal T-PTP and partition homogeneity test) shown that the data partitions are heterogeneous, which could be due to sampling errors (in either data set) or differences in the underlying phylogenies, and therefore data were not combined in a single analysis. ❐ *Porifera, Axinellidae, secondary structure, D3 domain 28S ribosomal DNA, phylogeny.*

B. Alvarez (email: b.glasby@niwa.cri.nz), M.D. Crisp, Division of Botany and Zoology, Australian National University, Canberra, ACT 0200, Australia; F. Driver, Division of Entomology, CSIRO, Canberra, ACT 2601, Australia; J.N.A. Hooper, Queensland Museum, P.O. Box 3300, South Brisbane, QLD, 4101, Australia; R.W.M. van Soest, Institute of Taxonomic Zoology, University of Amsterdam, P.O. Box 20125-1000 at Amsterdam, The Netherlands; * Present address: National Institute of Water and Atmospheric Research, P.O Box 14-901, Kilbirnie, Wellington, New Zealand;* *1 June 1998.*